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MUTAGENESIS TESTING WITH MAMMALIAN CELLS: VALIDATING AND ADAPTING A MULTIPLE-MARKER BIOASSAY TO ACTIVATE AND DETECT MUTAGENS IN CRUDE SAMPLES

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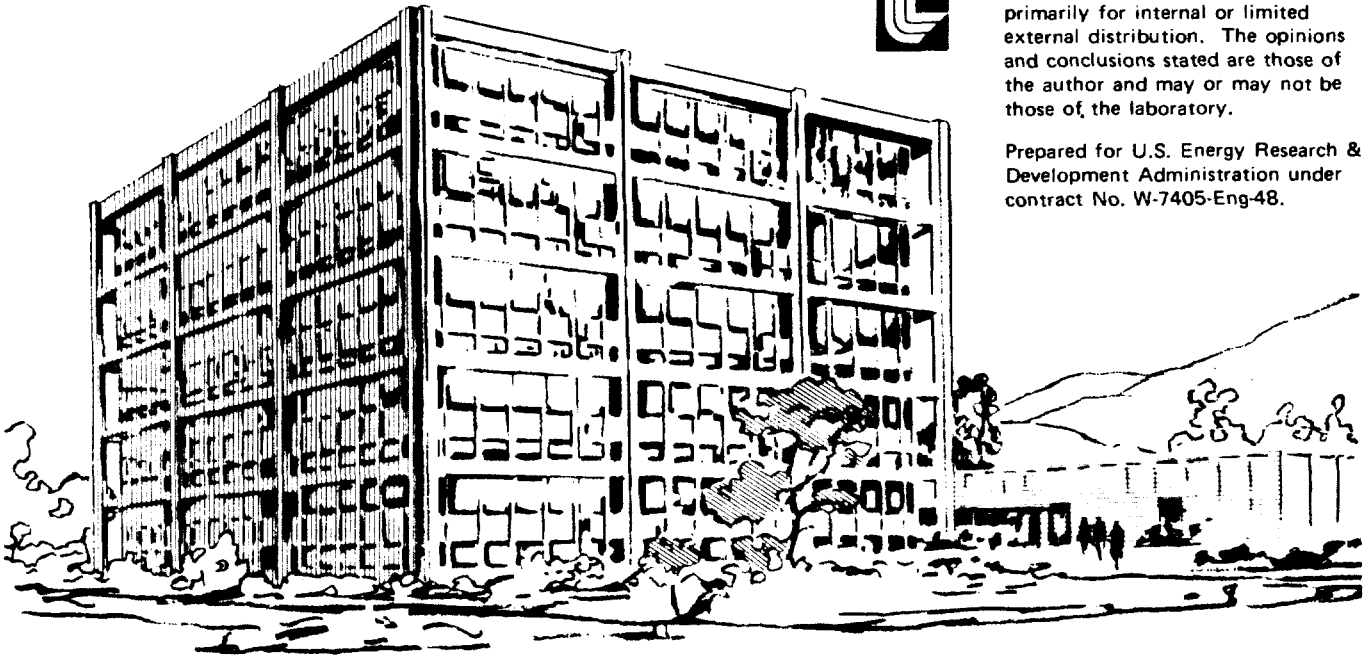

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MUTAGENESIS TESTING WITH MAMMALIAN CELLS: Validating and Adapting a Multiple-Marker Bioassay to Activate and Detect Mutagens in Crude Samples for Energy Technology

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Progress Report

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PROGRESS REPORT

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I. SUMMARY

Mutagenesis assays employing a system that can detect and quantitate forward mutation at multiple gene loci may be more efficient than single-locus assays in detecting a broad spectrum of potentially mutagenic agents. The Chinese hamster ovary (CHO) AT3-2 cell line described herein is functionally heterozygous in both the aprt (adenine phosphoribosyltransferase, EC 2.4.2.7) and tk (thymidine kinase, EC 2.7.1.75) genes. The AT3-2 cell line therefore can in a single step select mutants at four gene loci (aprt, tk, hgprt, ATPase) that represent X-linked, autosomal dominant, and autosomal recessive markers. There are indications that, in these hamster cells, use of the TK marker may detect chromosomal anomalies in addition to point mutation. Experiments are still in progress to determine the exact nature of the lesions contributing to the high observed frequency of stable, resistant mutant phenotypes at the tk locus. If the lesions should represent, in part, mitotic recombination or nondisjunction, the marker could be very sensitive for detecting certain compounds that produce gross chromosomal aberrations while producing relatively few point mutations. Thus, this marker may be a unique addition to the concept of multiple-marker assays for mutagen testing.

Because most pollutants of environmental concern are promutagens requiring metabolic activation, we have used a variety of techniques for in vitro metabolic activation (S-9 supernatant, concentrated microsomes, cell-mediated techniques with Syrian hamster embryo and primary rat hepatocytes) from induced and noninduced rodents. The optimal activation procedures can now be coupled with the Multiple-Marker Mutagenesis Assay (MMMA) and used to further develop and validate test procedures.

To extend in vitro activation to sources other than rat liver, we have used kidney microsomes from C3H/HeJ mice, a species where the male susceptibility to DMN-induced kidney tumors is higher than that of the female. Kidney microsomes from male and female mice were compared for their ability to activate DMN into mutagenic metabolites; preparations from male kidney yielded DMN-induced mutant frequencies 3 to 22 times higher at the four loci than control or female values. Thus, in vitro mutagenesis measured at multiple mammalian genes correlated with known

sex differences in whole animal tumor studies, where DMN was activated *in vivo*. This demonstrates the use of the MMMA with activation material from other species or organ sources and shows the feasibility of relating *in vitro* test results to sex-specific carcinogenesis *in vivo*.

Current and future plans include (1) participating in the ICI/MRC/NIEHS Collaborative Study, to test a minimum of ten unknowns in blind experiments; (2) testing several compounds that require activation and are potent frameshift mutagens in the Salmonella assay, i.e., quercetin, a plant flavonol, and two derivatives of pyrolyzed tryptophan that may be present in cooked food consumed by man; (3) validating the MMMA with chemicals of known activity, e.g., the anthracene series that require activation and are potent to varying degrees in whole-animal carcinogenesis studies. In addition, much work remains to adapt mammalian bioassays to accept crude mixtures and multiple-agent fractions. When this work is complete, the MMMA will be ready for broad application (a) in testing suspect agents for their cellular and genetic toxicity either alone or in combination as components of complex mixtures and (b) in evaluating the potential health effects associated with effluents from a wide variety of sources.

II. DETAILED PROGRESS FOR THE REPORTING PERIOD

A. Development and Validation of a Fourth Marker, Thymidine Kinase

Because the type of mutational damage and the mechanism by which it is incurred may vary considerably for different mutagens and carcinogens, specific genetic loci may differ markedly in their sensitivities to a given mutagen. Mutagenesis assay systems employing multiple markers may therefore be more effective in detecting a broad spectrum of potentially mutagenic agents. Our CHO-IB2 cell line, which is heterozygous at the autosomal recessive aprt locus, allows a simultaneous quantitation and direct comparison of the extent of mutational damage at three specific genetic loci (aprt, adenine phosphoribosyltransferase, EC 2.4.2.7; hgprt, hypoxanthine-guanine phosphoribosyltransferase, EC 2.4.2.8; Na^+-K^+-ATPase, EC 3.6.1.3). Efforts to develop a fourth genetic marker to include in our assay system have focused upon the tk locus (thymidine kinase, EC 2.7.1.75). Operationally, the relatively short expression time required for maximal expression of mutation at this locus (Clive et al., 1972; Skopek et al., 1978; Clive et al., 1979) makes it useful in mutagenesis assays. Furthermore, mutant validation and application of the L5178Y $TK^{+/-}$ mouse lymphoma system developed by Clive and co-workers (Clive et al., 1972; Clive and Voytek, 1977; Clive et al., 1979) makes it especially desirable to be able to use this marker in our CHO cell line, in which mutagenesis at this and three other gene loci (Fig. 1) can be compared directly.

Use of the autosomal recessive tk locus to assay mutagenesis first requires the derivation of a $tk^{+/-}$ heterozygote. With only one functional allele present, single-step forward mutation to resistance to thymidine analogues can be detected at reasonable frequencies. On the basis of the TK-specific activity of CHO-IB2, which was approximately one-half that of several other Chinese hamster cell lines (Table I), it initially seemed that IB2 might be fortuitously hemizygous at this locus. Further genetic analysis, including mutation experiments and segregation analysis, ruled out this possibility; CHO-IB2 cells seem to possess two functional alleles at the tk locus. We have demonstrated unstable, nonmutational expression of resistance to the thymidine

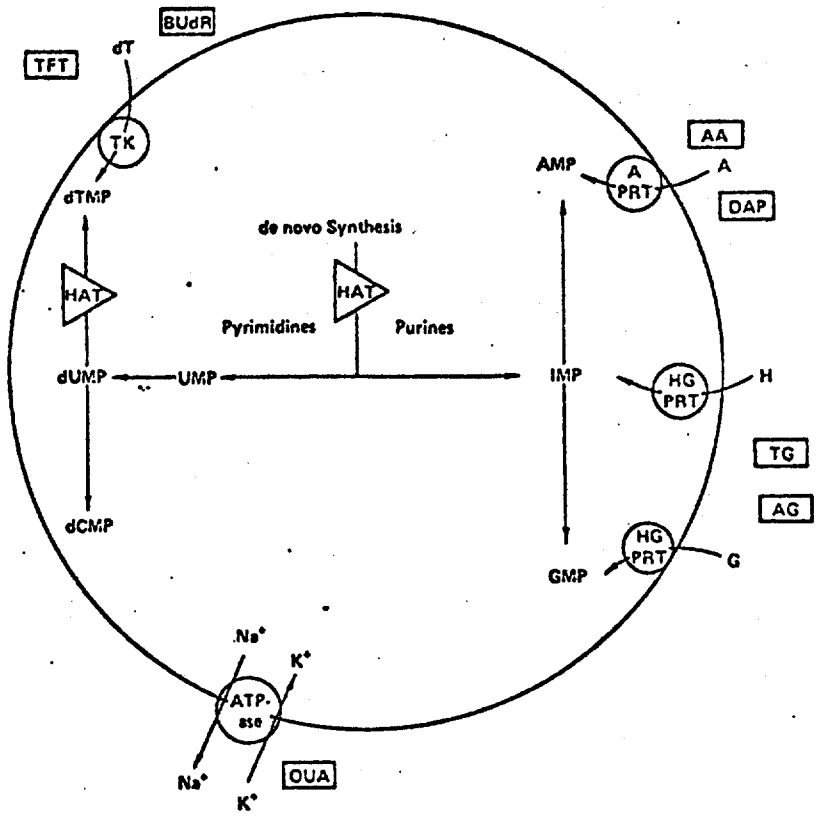


Fig. 1. Multiple-Marker Mutagenesis Assay (MMMA) allows direct comparison of mutagenesis data for four genetic markers (HGPRT, APRT, TK, ATPase). Abbreviations for selecting drugs are: AA, 8-azaadenine; AG, 8-azaguanine; BUdR, bromodeoxyuridine; DAP, diaminopurine; HAT, hypoxanthine-aminopterin-thymidine; OUA, ouabain; TFT, trifluorothymidine; TG, 6-thioguanine.

Table I. Thymidine kinase activity of Chinese hamster cell lines.

Cell line	Specific activity of thymidine kinase (pmol/min•mg protein)[a]	
CHO-K1	370 ± 28	(6)
SC1	353 ± 21	(6)
Gat^-	377 ± 22	(4)
RT-2	351 ± 19	(3)
C10A	169 ± 12	(8)
IB-2	171 ± 15	(27
IB-2, TFT^r isolates	159 ± 15	(28)

[a]Specific activity ± S.D. based on the number of times the assay was carried out (shown in parentheses).

analogue trifluorothymidine (TFT) in CHO-IB2 cells (Adair and Carver, preprint attached). However, we have been able to select a cell line which appears to be hemizygous at the tk locus and that can be employed for single-step selection of bona fide mutants that lack TK activity and are resistant to several cytotoxic thymidine analogues.

Although $aprt^{+/-}$heterozygotes can be readily isolated by their resistance to intermediate levels of selection drug, e.g., 8-azaadenine (Carver et al., 1978; Carver et al., submitted to Mutat. Res.), such an approach was unsuccessful for the TK marker. Direct selection of $tk^{+/-}$ heterozygotes in intermediate concentrations of 5-bromodeoxyuridine (BUdR) was impractical due to excessive background growth of wild-type cells. Attempts to isolate $tk^{+/-}$ heterozygotes using trifluorothymidine (TFT) yielded unstable drug-resistant variants at spontaneous frequencies $>10^{-5}$. Thus, we were forced to employ an indirect approach similar to that used by Clive et al (1972). As a first step to obtain a presumptive heterozygote, $tk^{-/-}$ mutants were selected from unmutagenized CHO-IB2 cell populations in a medium containing 100

μg/ml BUdR. The resistant ($BUdR^r$) colonies were recovered at a spontaneous frequency of 1.4×10^{-7} (eight mutant colonies out of 5.8×10^7 viable cells plated). The colonies were isolated, grown to mass culture, and assayed for TK activity. All eight spontaneous $BUdR^r$ strains as well as 32/32 mutagen-induced $BUdR^r$ or $FUdR^r$ isolates had barely detectable levels ($\leqslant$1% wild type) of TK activity (Table II). The $BUdR^r$, $FUdR^r$, or HAT^s (Hat-sensitive) phenotypes of these TK^- isolates were stable during several months of continuous culture, with little or no accumulation of spontaneous revertants.

To obtain $\underline{tk}^{+/-}$ heterozygotes, eight $BUdR^r$ TK^- strains derived from unmutagenized CHO-IB2 cell populations were treated with ethyl methanesulfonate (EMS) and plated into hypoxanthine-aminopterin-thymidine (HAT) medium to select for HAT^r, $\underline{tk}^{+/-}$ revertants. Five of the eight TK^- strains yielded HAT^r revertants after EMS treatment; revertant frequencies ranged from $< 3 \times 10^{-7}$ to 5.7×10^{-5} (Table III). A total of 33 HAT^r revertants were isolated, grown to mass culture, and assayed for TK activity. Specific activities of these revertants ranged from 7 to 184 pmol/min•mg protein, compared to 171 $\pm$ 15 for wild type CHO-IB2 cells and $\sim$2 pmol/min•mg protein for the TK^- mutants; most of the revertants had 10 to 30% of wild type activity (Table III).

Further characterization of several presumptive heterozygotes with intermediate levels of TK activity (35 to 50% of wild type) differed in their $\underline{in\ vivo}$ thermolability and substrate concentration optima for TK. Biochemical characterization of one such revertant, designated AT3-2, is summarized in Table IV. The altered kinetic and physical properties of TK in crude extracts of AT3-2 suggest that this revertant may reflect a second site, intragenic suppressor mutation at the structural gene locus of TK. The altered thermolability (Table IV) and biphasic thermal inactivation kinetics of the revertant enzyme (Fig. 2) indicate the probable presence of more than one form or species of TK activity. This is precisely what would be expected in the case of a second site, intragenic suppressor mutation of one of two mutant alleles of the TK locus, with subsequent expression of both gene products.

TABLE II.

Thymidine kinase activities of thymidine-analogue-resistant mutants from wild type or presumptive TK-heterozygous cells.

$$\left[\text{CHO-IB2} \xrightarrow[\text{Spont}]{\text{BUdR}^r} \text{SBU}^r\text{-3} \xrightarrow[\text{EMS}]{\text{HAT}^r} \text{SBU}^r\text{-3-ER2 (AT3-2)}\right]^a$$

Parental cell line	Selection agent	Spontaneous frequency[b]	No. mutants assayed		No. with ⩽1% wild-type TK activity	Other
			Spontaneous	Induced		
C10A-19	BUdR	9.0×10^{-7} (2)	0	3	3	0
($tk^{+/+}$)	FUdR	$<5.0 \times 10^{-7}$	0	3	3	0
CHO IB2	BUdR	1.4×10^{-7} (8)	8	18	26	0
($tk^{+/+}$)	FUdR	6.0×10^{-8} (1)	1	8	9	0
Stable	TFT	$<4.0 \times 10^{-7}$	0	2[c]	2	0
Unstable	TFT	2.4×10^{-5}	14	14	0	28 (~Wild type)
SBU^r-3-ER2	BUdR	2.8×10^{-3}	6	0	6	0
(AT3-2)	FUdR	2.0×10^{-3}	6	0	6	0
($tk^{+/-}$)	TFT	1.9×10^{-3}	6	0	5	1 (~15% Wild type)

[a]Derivation of presumptive TK-heterozygous cells. A spontaneous TK^- mutant fully resistant to BUdR, i.e., SBU^r-3, was selected from wild type CHO-IB2 and mutagenized with ethyl methanesulfonate (EMS); the mutagenized population was then placed into HAT medium to select a resistant (HAT^r) presumptive heterozygote (SBU^r-3-ER2). The strain cloned from SBU^r-3-ER2 was designated AT3-2.

[b]Numbers in parentheses refer to total number of colonies determining frequencies

[c]0/96 Spontaneous and 2/38 mutagen-treated were stable upon test

Table III. Analysis of HAT^r revertants (presumptive $tk^{+/-}$ heterozygotes).

Parental cell line	Frequency of HAT^r revertants	Revertants assayed	Distribution of revertant thymidine kinase activities (% wild-type activity)			
			<10	10-30	30-70	70-100
SBU^r-1	2×10^{-7} (spontaneous)	1	0	0	0	1
	4.4×10^{-6} (EMS)	12	4	4	2	2
SBU^r-2	5.7×10^{-5} (EMS)	4	2	1	1	0
SBU^r-3	$<1 \times 10^{-7}$ (spon)	0	-	-	-	-
	3.1×10^{-5} (EMS)	4	0	3	1	0
SBU^r-4	$<3 \times 10^{-7}$ (EMS)	0	-	-	-	-
SBU^r-5	$<3 \times 10^{-7}$ (EMS)	0	-	-	-	-
SBU^r-6	3.6×10^{-5} (EMS)	4	0	4	0	0
SBU^r-7	3.0×10^{-5} (EMS)	4	0	4	0	0
SBR^r-8	8.3×10^{-6} (EMS)	4	1	3	0	0
TOTALS		33	7	19	4	3

Table IV. Analysis of $tk^{+/-}$ heterozygous stock line AT3-2.

Cell line	Specific Activity (pmol/min·mg protein)[a]	Thermolability $t_{1/2}$ at 45°C (min)	Km dT	Optimal ATP
IB-2	171 ± 15 (27)	42.5 min	3-4 μM	2-5 mM
SBU^r-3-ER2 (AT3-2)	61 ± 6 (10)	26.5 min	2-3 μM	10-15 mM

Cell line	Relative TK activity with inhibitor				Relative TK activity with		
	40 M dTTP	40 M BUdR	40 M FUdR	40 M TFT	5 mM ATP	5 mM AMP	5 mM CTP
IB-2	0.819	0.638	0.193	0.487	1.000	0.003	0.104
SBU^r-3-ER2 (AT3-2)	0.839	0.561	0.139	0.436	1.000	0.001	0.083

[a]Specific activity ± S.D. based on the number of times assayed (shown in parentheses).

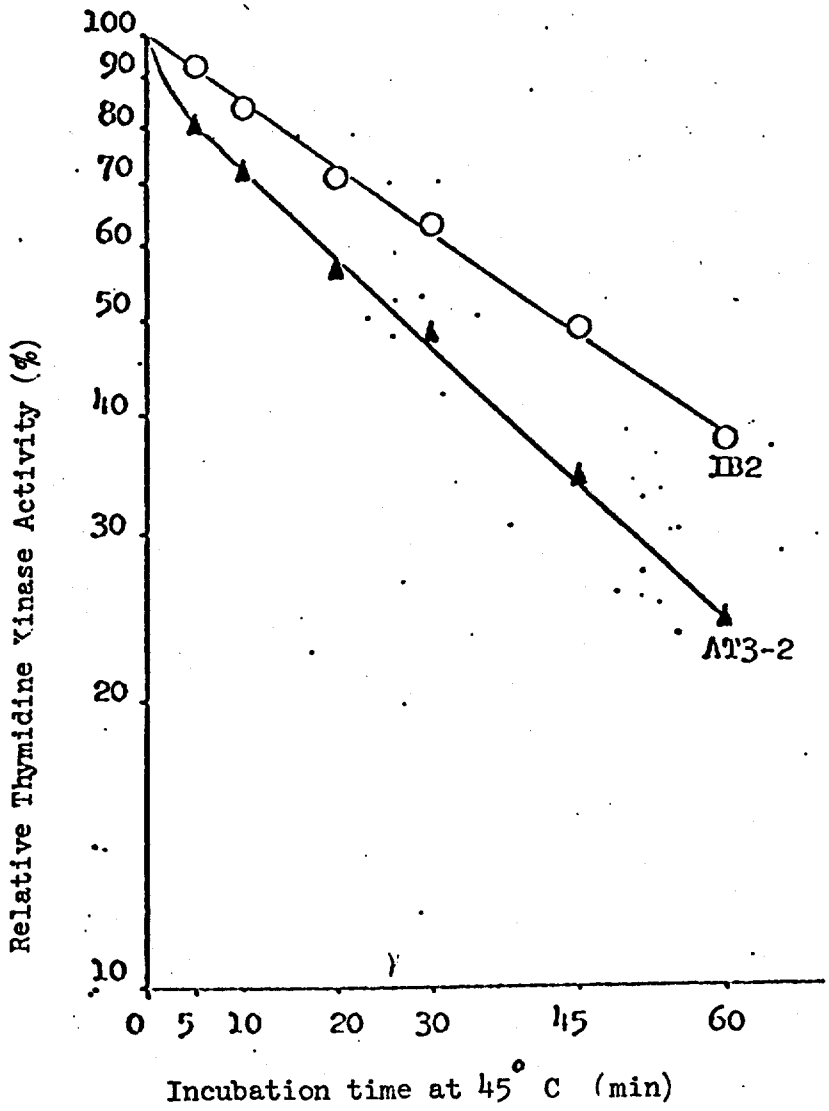


Fig. 2. Thermal inactivation of thymidine kinase (TK) in extracts of CHO-IB2 cells (o) and the presumptive $tk^{+/-}$ heterozygote, AT3-2 (▲). Aliquots of each extract were incubated at 45°C for the time indicated, chilled on ice, and subsequently assayed at 37°C for remaining TK activity. Specific activities for TK in unheated extracts of CHO-IB2 and AT3-2 were 159.8 and 56.1 pmol/min mg, respectively.

Table V. Segregation analysis of AT3-2 for expression of AA^r and $BUdR^r$ phenotypes.

	Frequency of drug-resistant segregant colonies		
	TG^r	AA^r	$TG^r \cdot AA^r$ [a]
AA^r mutant X wild type (IB2)			
$GAMA^r$ hybrid H-1	4.3×10^{-3}	5×10^{-6}	---
$GAMA^r$ hybrid H-2	4.7×10^{-3}	7×10^{-6}	---
AA^r mutant X presumptive heterozygote (AT3-2)			
$GAMA^r$ hybrid H-1	6.4×10^{-3}	1.4×10^{-3}	2×10^{-5}
$GAMA^r$ hybrid H-2	7.6×10^{-3}	6.8×10^{-4}	1×10^{-5}

	Frequency of drug-resistant segregant colonies		
	TG^r	$BUdR^r$	$TG^r \cdot BUdR^r$ [a]
$BUdR^r$ mutant X wild type (IB2)			
HAT^r hybrid H-1	5.8×10^{-3}	4×10^{-7}	---
HAT^r hybrid H-2	4.0×10^{-3}	1×10^{-6}	---
$BUdR^r$ mutant X presumptive heterozygote (AT3-2)			
HAT^r hybrid H-1	4.8×10^{-3}	2.5×10^{-4}	5×10^{-6}
HAT^r hybrid H-2	6.4×10^{-3}	5.0×10^{-4}	7×10^{-6}

[a] Frequency of mutants selected simultaneously with TG and AA (or BUdR) and resistant to both drugs.

The heterozygous nature of AT3-2 was further tested by forward mutation experiments and segregation analysis. Although spontaneous $BUdR^r$ or $FUdR^r$ (TK^-) colonies were rarely recovered from the $tk^{+/+}$ CHO-IB2 line, such colonies were obtained from AT3-2 cell populations at a spontaneous frequency of approximately 2×10^{-3} (Table II). Segregation analysis of AT3-2 confirmed this strain of CHO cells to be heterozygous at both the aprt and tk loci (Table V).

However, the extraordinarily high frequency of $tk^{-/-}$ mutants in unmutagenized populations of AT3-2 was 2 to 4 orders of magnitude greater than those typically observed at other genetic loci. Similar frequencies for spontaneous forward mutation at the tk locus were reported previously for presumptive $tk^{+/-}$ heterozygotes of V79 cells (Roufa et al., 1973), but whether the high spontaneous frequencies reflected actual mutation rates was not clear from these studies. Our results from Luria-Delbruck fluctuation analyses of BUdR-, FUdR-, and TFT-resistance in AT3-2 cells (Table VI) indicated that the high mutant frequencies were probably the result of an unusually high apparent rate of spontaneous mutation at the tk locus in the heterozygote. The estimated mutation rate is approximately three orders of magnitude higher than those for single-step forward mutation at the HGPRT or APRT markers in the same cell line, and may reflect either a "hot spot" for mutation at the tk locus in AT3-2 or possible extra mutational events such as mitotic recombination or nondisjunction. Complementation analysis of $BUdR^r$, $FUdR^r$, or TFT^r, TK^- derivatives of AT3-2 (and the parental TK^- SBU^r-3 strain) indicated that these TK^- mutants behave as recessives, failing to complement either each other or other TK^- Chinese hamster lines such as DON A3 (Westerveld et al., 1971) or V79 (462-10) (Roufa et al., 1973) (Table VII). These results indicate that all of the above strains belong to a single TK-complementation class. The TK-deficient DON A3 cell line was previously used to map the tk locus in the mouse (McBreen et al., 1977; Kozak and Ruddle, 1977) and human (Orkwiszewski et al., 1974; Elsevier et al., 1974) karyotypes.

In summary, we have been able to select for a cell line that is functionally hemizygous at both the aprt and tk genes and that can be employed for single-step selection of forward, autosomal recessive

Table VI. Luria-Delbruck fluctuation analyses of thymidine analogue resistance in AT3-2 cells.

	$BUdR^r$		$FUdR^r$		TFT^r	
Replicate cultures	31	1	31	1	31	1
Initial cell number per replicate	49	49	49	49	49	49
Final cell number per replicate	2.7×10^7	2.7×10^7	2.7×10^7	2.7×10^7	2.7×10^7	2.7×10^7
Cells per sample; no. of samples	9.0×10^4(1)	3.0×10^4(12)	9.0×10^4(1)	3.0×10^4(12)	9.0×10^4(1)	3.0×10^4(12)
Number of mutants per sample						
Range	138-293	48-70	83-225	37-50	77-205	33-47
Median	180	58	124	40	127	39
Mean	188.6	57.7	134.6	40.9	130.4	40.0
Variance	1597	28.2	1266	35.7	852	22.9
Ratio: variance/mean	8.47	0.49	9.41	0.87	6.53	0.57
Chi-square	254	5.23	282	9.60	183	6.30
P	$\ll 0.001$	>0.90	$\ll 0.001$	>0.60	$\ll 0.001$	>0.80
Mutation rate calculated by						
Mean method[a]	$2.1 \pm 0.5 \times 10^{-4}$/cell·gen		$1.6 \pm 0.4 \times 10^{-4}$/cell·gen		$1.5 \pm 0.3 \times 10^{-4}$/cell·gen	
Median method[b]	$3.5 \pm 0.4 \times 10^{-4}$/cell·gen		$2.6 \pm 0.3 \times 10^{-4}$/cell·gen		$2.6 \pm 0.3 \times 10^{-4}$/cell·gen	
Accumulation with time[c], exp A	2.7×10^{-4}/cell·gen		1.8×10^{-4}/cell·gen		1.8×10^{-4}/cell·gen	
exp B	1.7×10^{-4}/cell·gen		1.2×10^{-4}/cell·gen		1.2×10^{-4}/cell·gen	
Theoretical variance	7590		4060		3410	
Standard deviation						
Experimental	0.212		0.265		0.224	
Theoretical	0.460		0.472		0.447	
Poisson	0.073		0.086		0.088	

a Luria & Delbruck, 1943.

b Lea & Coulson, 1948.

c Shapiro et al, 1972; Experiments A and B separated by three additional days of expression time.

TABLE VII

Complementation analysis of TK-deficient Chinese hamster cell lines

	DON A3	V79 (462-10)	SBU^r-1	SBU^r-2	SBU^r-3	ETFT^r-2	SBUdR^r-1	STFT^r-1	STG^r-1
DON A3	-	-							
V79 (462-10)	-	-							
SBU^r-1	-	-	-						
SBU^r-2	-	-	-	-					
SBU^r-3	-	-	-	-	-				
ETFT^r-2	-	-	-	-	-	-			
AT3-SBUDR^r-1	-	-	-	-	-	-	-		
AT3-STFT^r-1	-	-	-	-	-	-	-	-	
STG^r-1	+	+	+	+	+	+	+	+	-

mutants at both loci. The $tk^{+/-}$ heterozygote has approximately 40% of the wild-type TK activity and was obtained by EMS mutagenesis, i.e., back mutation, of a fully-deficient mutant. Inclusion of this marker in the Multiple-Marker Mutagenesis Assay will allow the measurement of mutation at four gene loci (hgprt, aprt, tk, ATPase) representing both X-linked and autosomal markers. Currently, the TK marker is used extensively and almost exclusively in the mouse lymphoma (L5178Y) system; the mouse lines are heteroploid to varying degrees, but the CHO cell line AT3-2 is near diploid. Incorporation of the TK marker into our hamster assay system will allow valuable data for species comparison and provide the multiple-marker assay with a second autosomal recessive gene locus for mutagenesis testing. Experiments are currently in progress to determine the nature of the lesions that cause the high spontaneous and induced TK^- mutant frequencies. If they do represent mitotic recombination or other chromosomal anomalies, the marker could be very sensitive for certain classes of compounds that are relatively inefficient at producing point mutation. Thus, this marker may represent a unique addition to the concept of multiple-marker mutagenesis assays to test mutagens.

B. Opimizing Activation Techniques to be Coupled with the Multiple-Marker Mutagenesis assay

Most environmental pollutants are promutagens that require metabolic activation; mutagenesis assays currently in use rely on in vitro activation by microsomal homogenates or metabolically-competent cultured cells. To determine the optimal parameters for our Chinese hamster ovary (CHO) system, we have conducted pilot experiments with:

- Aroclor-induced rat liver S-9;
- Concentrated microsomes from Arocolor-induced rat liver;
- Concentrated microsomes from mouse kidney (male, female, testosterone-treated female);
- Cell-mediated activation by Syrian hamster embryo (SHE) cells;
- Activation by feeder layers of primary rat hepatocytes (HEP).

The above experiments were designed to compare the relative efficiences of microsomal vs. S-9 activation, compare cell-free in vitro techniques with cell-mediated activation, and gain experience with alternate rodent sources, i.e., mouse kidney. We initially intended to

use commercially obtained S-9 material. However, the available preparations suffered from a lack of quality control (vial-to-vial variations ranging up to 100% in total protein concentration). Thus, we were forced to delay experiments and develop procedures to process the liver homogenates in our laboratory. Induced rat liver material was prepared as the S-9 fraction (supernatant from 9,000 x g centrifugation) and concentrated microsomal fraction (pellet from 100,000 x g centrifugation). The response from the activation material prepared in our laboratory was similar to that obtained with the commercial S-9 except that the heterogeneity of commercial samples was successfully avoided enabling reproducible experiments.

1. Comparisons of rat liver in vitro metabolic activation with cell-mediated techniques.

In Fig. 3a, the S-9 and microsomal activation of benzo(a)pyrene (BP) and dimethylnitrosamine (DMN) are given as the observed mutant frequency at four gene loci vs relative survival; the latter is proportional to applied mutagen dose and is considered to be a biological measure of the effective mutagen dose achieved over the brief 2 h period of in vitro activation. At the concentrations used (1.1 to 1.6 mg/ml), the S-9 fraction plus cofactor mix did not significantly increase mutagenesis at any of the four loci after exposure with BP at 10 to 15 μg/ml. Experiments in progress indicate that BP-induced mutants are obtained with lower concentrations of S-9 (ca 0.8 mg/ml) and 10 μg/ml BP, but the efficiency of S-9 to activate BP is lower than that of concentrated microsomes. With S-9 concentrations ranging from 1.1 to 1.6 mg/ml, the mutant frequency after DMN treatment (10 mM) was greatly enhanced at all four loci. Survival was low ($\leq$10%) and additional data are being obtained. Activation with a concentrated microsome fraction (concentrations of protein $\sim$ 0.25 to 0.75 mg/ml) increased BP mutagenesis (10 to 15 μg/ml), but the dose response was very nonlinear at all loci. Mutant frequencies with DMN (10 mM) in the presence of microsome protein and cofactors were increased at very low survival ($\leq$10%); additional experiments are in progress.

COMPARISON OF ACTIVATION SYSTEMS FOR BP- AND DMN-INDUCED MUTANTS IN CULTURED MAMMALIAN CELLS

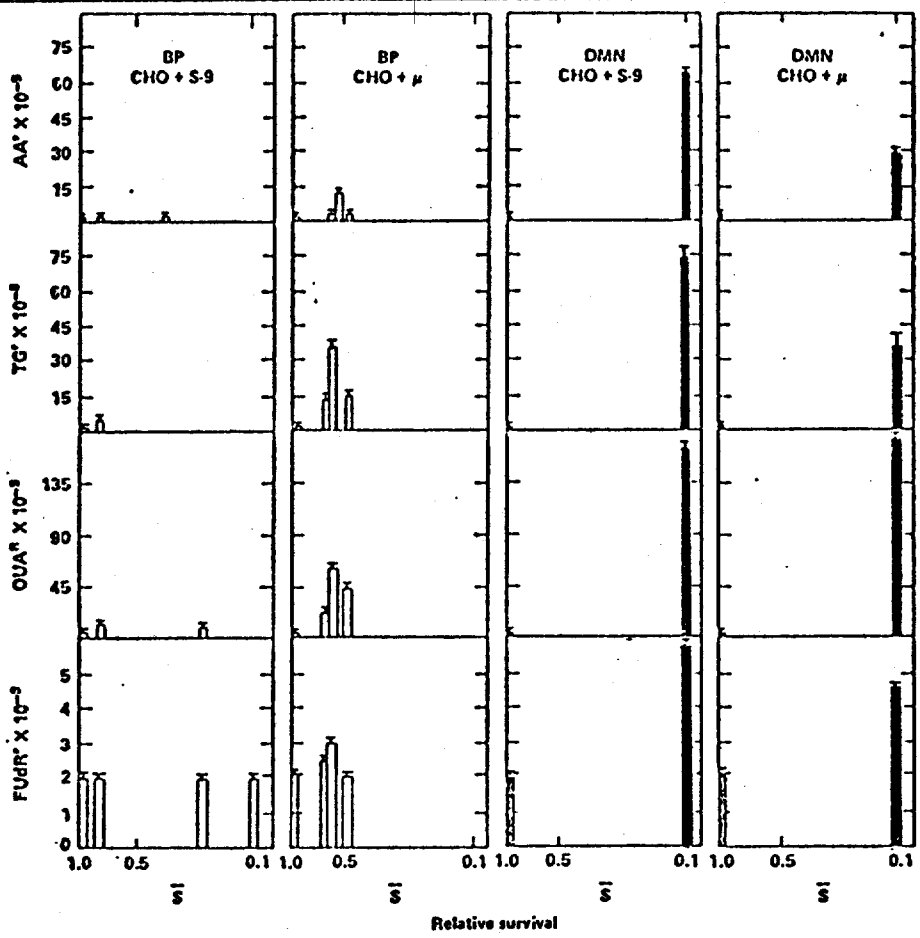


Fig. 3a. Activation of benzo(a)pyrene (BP) and dimethylnitrosamine (DMN) by S-9 or microsomes (μ). Induced mutation is shown at four gene loci (*aprt*$^{-/-}$, resistance to azaadenine, AA^r; *hgprt*$^-$, resistance to 6-thioguanine, TG^r; Na-K-ATPase, resistance to ouabain, OUA^R; and *tk*$^{-/-}$, resistance to fluorodeoxyuridine, FUdR^r). The ordinate quantitates the observed mutant frequency at each locus, shown as a function of the relative cell survival ($\bar{S}$) following mutagenesis with BP or DMN. Bars shown at high survival, e.g., 0.9 to 1.0, represent the mean of the spontaneous mutant frequency of untreated cultures, negative controls containing mutagen but no activation mix, and controls containing S-9 activation mix but lacking the mutagen. Error limits represent one standard error of the mean, SEM. The concentration of DMN was 10 mM; BP concentrations ranged from 10 to 15 μg/ml.

COMPARISON OF ACTIVATION SYSTEMS FOR BP- AND DMN- INDUCED MUTANTS IN CULTURED MAMMALIAN CELLS

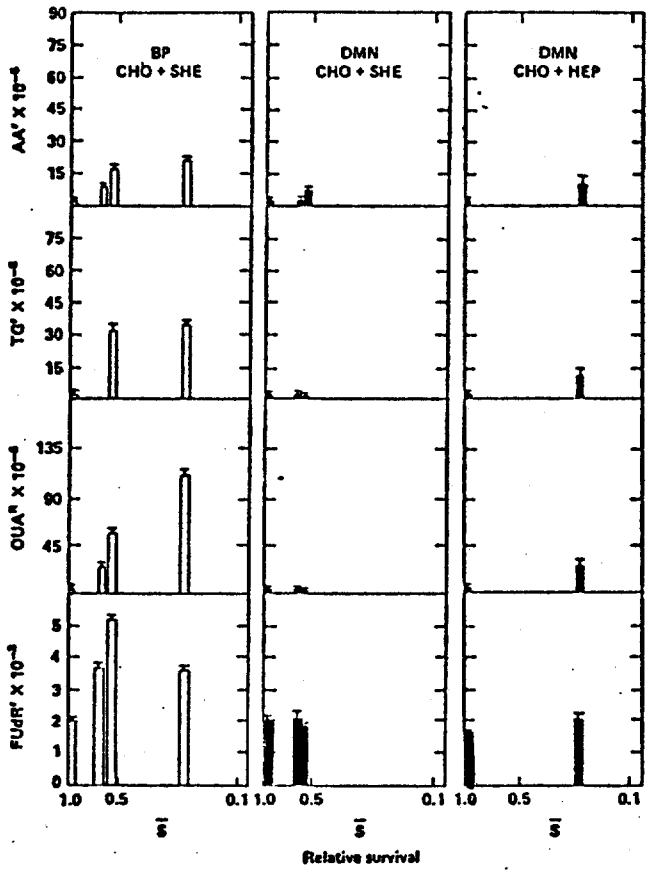


Fig. 3b. Cell-mediated activation of BP and DMN. Induced mutation is shown at four gene loci. The ordinate and abscissa are the same as in Fig. 3a, with spontaneous frequencies and error limits as previously defined. Activation by primary cultures of Syrian hamster embryo (SHE) cells and rat hepatocytes (HEP) of BP (2-10 μg/ml) and DMN (10 mM) to mutagenic metabolites is shown.

In Fig. 3b, mutagen-activation by cell-mediated techniques using feeder layers of SHE or HEP is shown. The feeder layers were combined with Chinese hamster ovary indicator cells to determine the mutant induction by BP and DMN. The procedure for obtaining rat hepatocytes is given in Fig. 4. Significant increases in induced mutant frequency were obtained at all four loci with BP (2 to 10 μg/ml) activated by SHE cells. The SHE cell preparation was not competent to activate DMN at 12 to 60 mM; however, the mutation induction by rat hepatocytes with DMN at 10 mM was increased above controls at 3 out of the 4 loci ($FUdR^r$ was not significantly enhanced). The rat hepatocyte experiments are technically difficult; we have made several attempts without significant success to optimize conditions for securing hepatocytes in liquid nitrogen. Therefore, the mutagenesis protocols require fresh, sterile, liver-perfusion material and at the current state of the art, this requirement is not considered practical for large-scale screening assays. For example, the experiment with rat-hepatocyte activation of BP was not sterile and mutagenesis data were not obtained. Very recent attempts to secure hepatocytes have involved incubation in DMSO at room temperature for longer periods, i.e., up to 1 h; the cells were then frozen at controlled freezing rates, with subsequent recovery of about 25%. We do not know at this time what the metabolic viability of these previously frozen cells might be. In general, we feel hepatocytes are more useful for basic studies of biochemical mechanisms than for application to screening assays. The SHE cells are easily obtained and frozen at very early passage, where they retain metabolic activity for a variety of compound classes. However, SHE cells cannot activate liver carcinogens such as DMN, as is apparent in Fig. 3b. The use of SHE cells as activating feeder cells thus is limited in mutagenesis testing of unknowns or of complex mixtures.

2. Activation Experiments with Mouse Kidney Microsomes

The use of in vitro preparations of microsomal proteins offers many advantages, e.g., they avoid the need to use whole animals for in vivo activation and they enable studies on differences in metabolic activity among organs. Many factors affect the sensitivity of in vitro techniques, including purity and amount of the microsome fractions used,

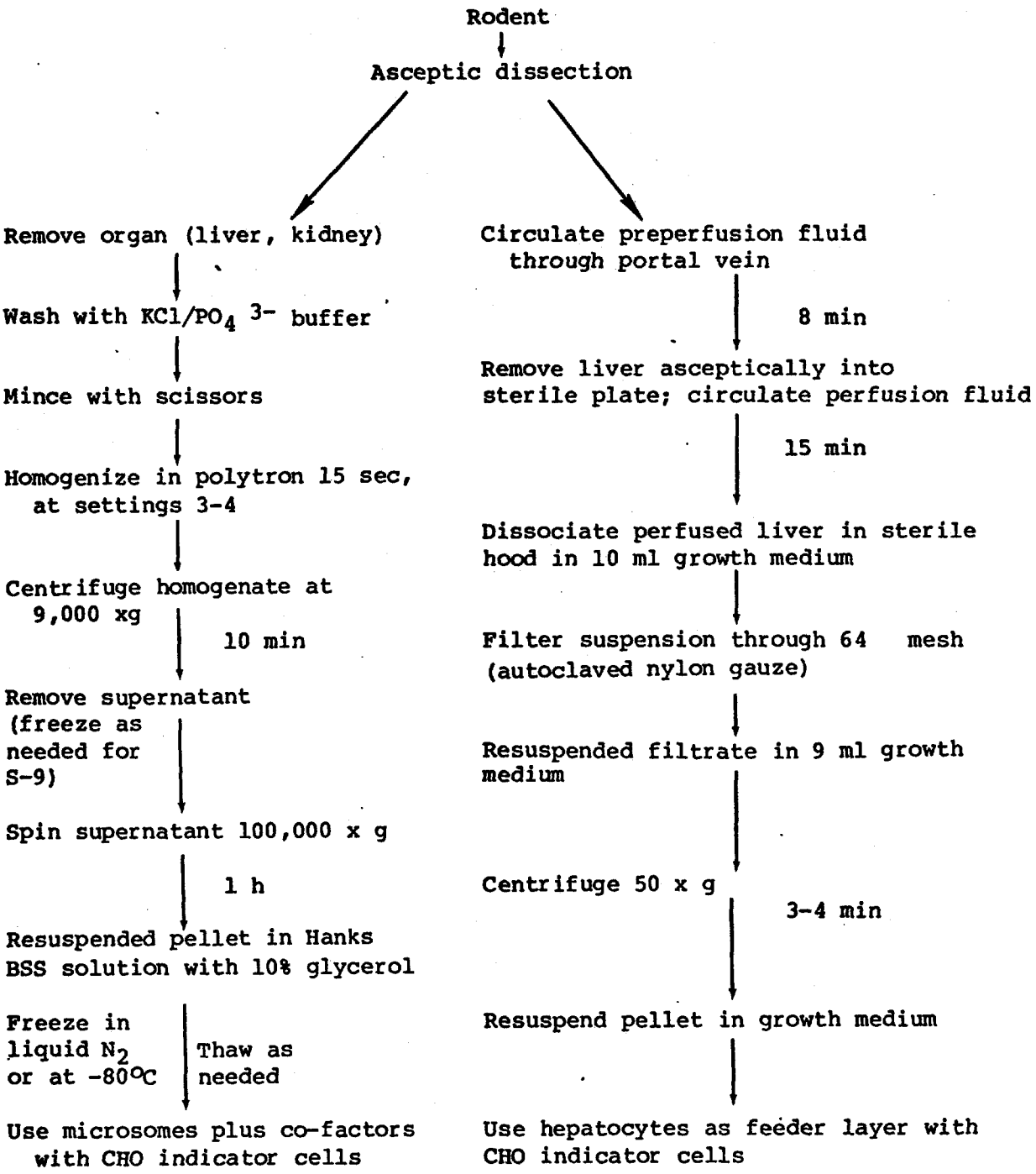


Fig. 4. Aseptic preparation of microsomes and hepatocytes.

the organ from which cells are derived, as well as sex and species differences. Brusick and co-workers (1977) compared the relationship between target-organ susceptibility of DMN-induced tumors and *in vitro* metabolic activation of DMN to a mutagen and found species, sex, and organ differences. To extend *in vitro* activation to another rodent and organ source, we chose the C3H/HeJ mouse, a species in which susceptibility of males to DMN-induced tumors of the kidney is much higher than that of females; the risk of DMN-induced liver tumors is apparently similar in both sexes, suggesting no differences in metabolism of DMN in the liver. We initiated experiments to determine the ability of C3H/HeJ mouse kidney microsomes to activate DMN into mutagenic metabolites, measuring forward mutation frequencies at the four drug-resistance markers in the CHO multiple-marker mutagenesis assay.

In Fig. 4, the procedure of preparing mouse kidney microsomes is given. In Fig. 5a, activation by kidney microsomes from the male and female mouse is compared; the observed mutant frequency induced by DMN is presented as a function of the relative cell survival after DMN treatment. Significant increases in mutant frequencies at all four loci were obtained in DMN-induced mutagenesis after metabolic activation by male microsomes at protein concentrations of 0.5 and 1.0 mg/ml. Activation by kidney microsomes from the female was insignficant; only the AA^r APRT marker showed a small but significant increase in DMN activated by female microsomes and occurred only at 1.0 mg/ml. All other conditions and markers yielded responses that were not significantly different from the controls. The implication that the female preparation contains a possible base-line activity of DMN activation will be difficult to confirm, because the observed mutant frequencies at all four loci decrease to control levels at microsomal protein concentrations of 2 mg/ml (presumably because of overall protein concentration influencing interactions with the active metabolites).

In Fig. 5b, the response to activation by kidney microsomes from male mice is compared to that from females treated with testosterone. At cell survivals $\leq 10\%$, preparations from male kidney yielded DMN-induced mutant

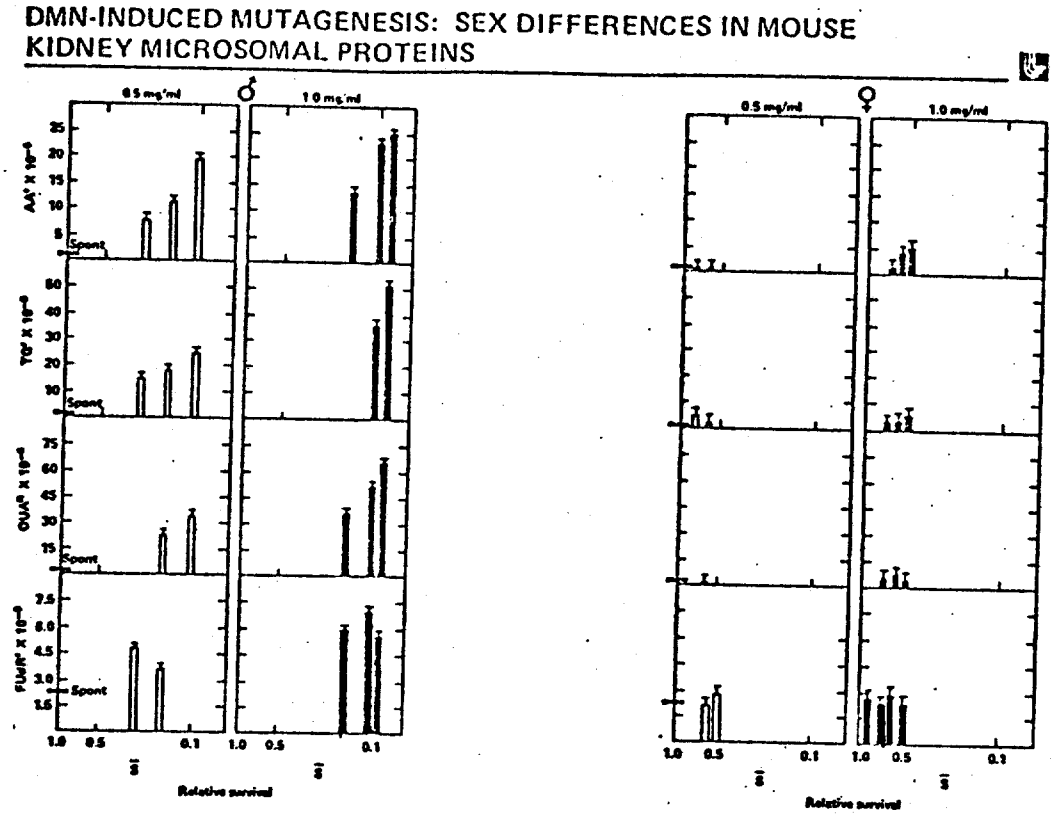


Fig. 5a. DMN-induced mutagenesis with activation by male or female kidney microsomes at microsomal protein concentrations of 0.5 mg/ml (open bars) and 1.0 mg/ml (closed bars). The error limits represent one SEM. Concentration of DMN ranged from 60 to 100 mM. The horizontal bar on the abscissa represents the mean of the spontaneous mutation frequency of untreated cultures and negative controls (i.e., DMN without activation and microsomes plus co-factors without DMN).

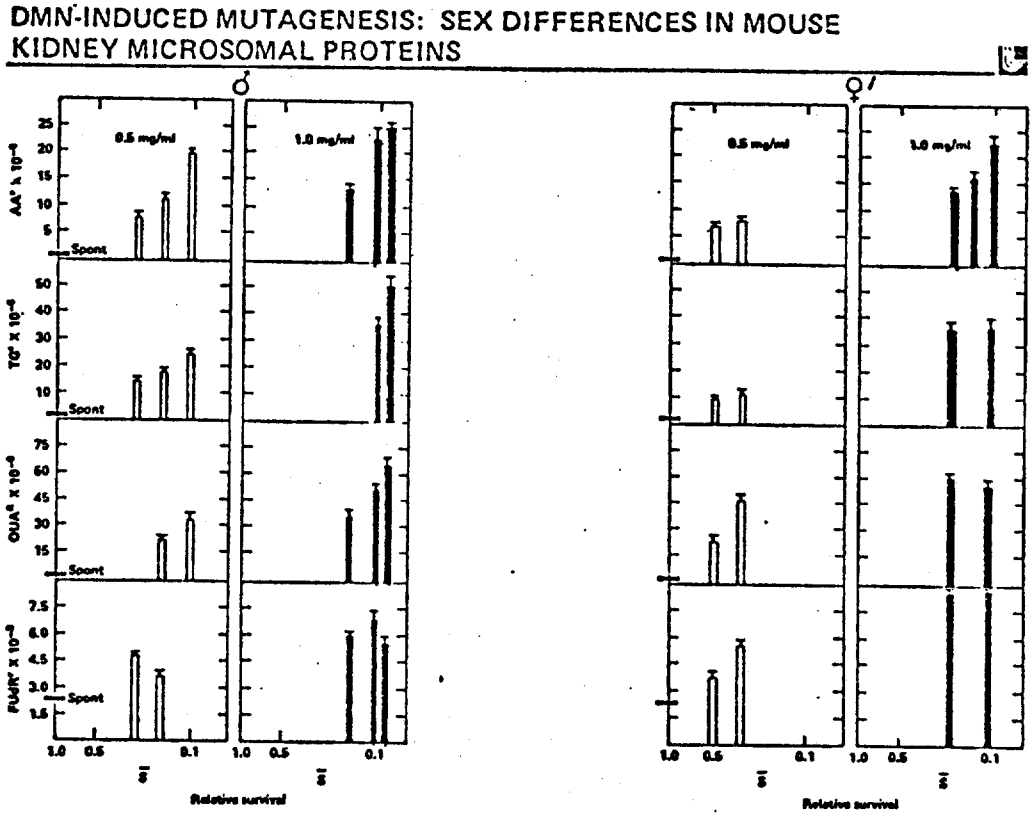


Fig. 5b. DMN-induced mutagenesis with activation by male or testosterone-treated female kidney. All parameters and symbols are as in Fig. 5a.

frequencies approximately 3 to 22 times higher at the four loci than control or female preparations; kidney from the testosterone-treated females increased DMN-induced mutation from 4 to 20 times at survivals of at least 10%.

In Fig. 6, more detailed responses of DMN activation by male, female, or testosterone-treated female microsomes are shown for the four loci in the multiple-marker mutagenesis assay. The observed mutant frequency per viable cell is shown as a function of the effective dose of DMN + microsomes that resulted in the relative cell survivals as shown. The applied concentrations of DMN ranged from 60 to 100 mM. The responses fit a linear model, with no significant difference between the data of microsomes from male and testosterone-treated female mice. The slope of the frequency curve of mutations induced by microsomes from the nontreated female at the aprt locus, AA^r, is significantly greater than zero at a $P \leq 10^{-4}$. The other three loci occasionally showed mutations somewhat above the range of negative controls and untreated cultures, but did not increase significantly with decreasing relative survival.

3. Summary and Conclusions from the Metabolic Activation Data

After the initial development of the Multiple-Marker Mutagenesis Assay and validation with direct mutagens, priority was given to determining which of the available metabolic activation techniques would be most sensitive, reliable, and generally applicable. We have completed preliminary experiments and have others in progress to optimize conditions for activation by rat liver S-9 fractions and microsomes and to compare the results with activation by intact cells (cell-mediated feeder-layer techniques). An alternative type of microsomal activation (mouse kidney) was used in experiments with DMN to broaden our experience and capability.

Experiments now being analyzed have determined the optimal concentrations of rat liver S-9 and microsomal protein (expressed as total protein) for mutant induction by BP and DMN. Other compounds (and compound classes) may require additional optimization of protein concentrations. It seems that both S-9 fractions and microsomes can activate BP and DMN, although at somewhat disparate concentrations (S-9 fraction ~ 0.8 to 1.6 mg/ml and microsomes ~ 0.25 to 0.75 mg/ml). In

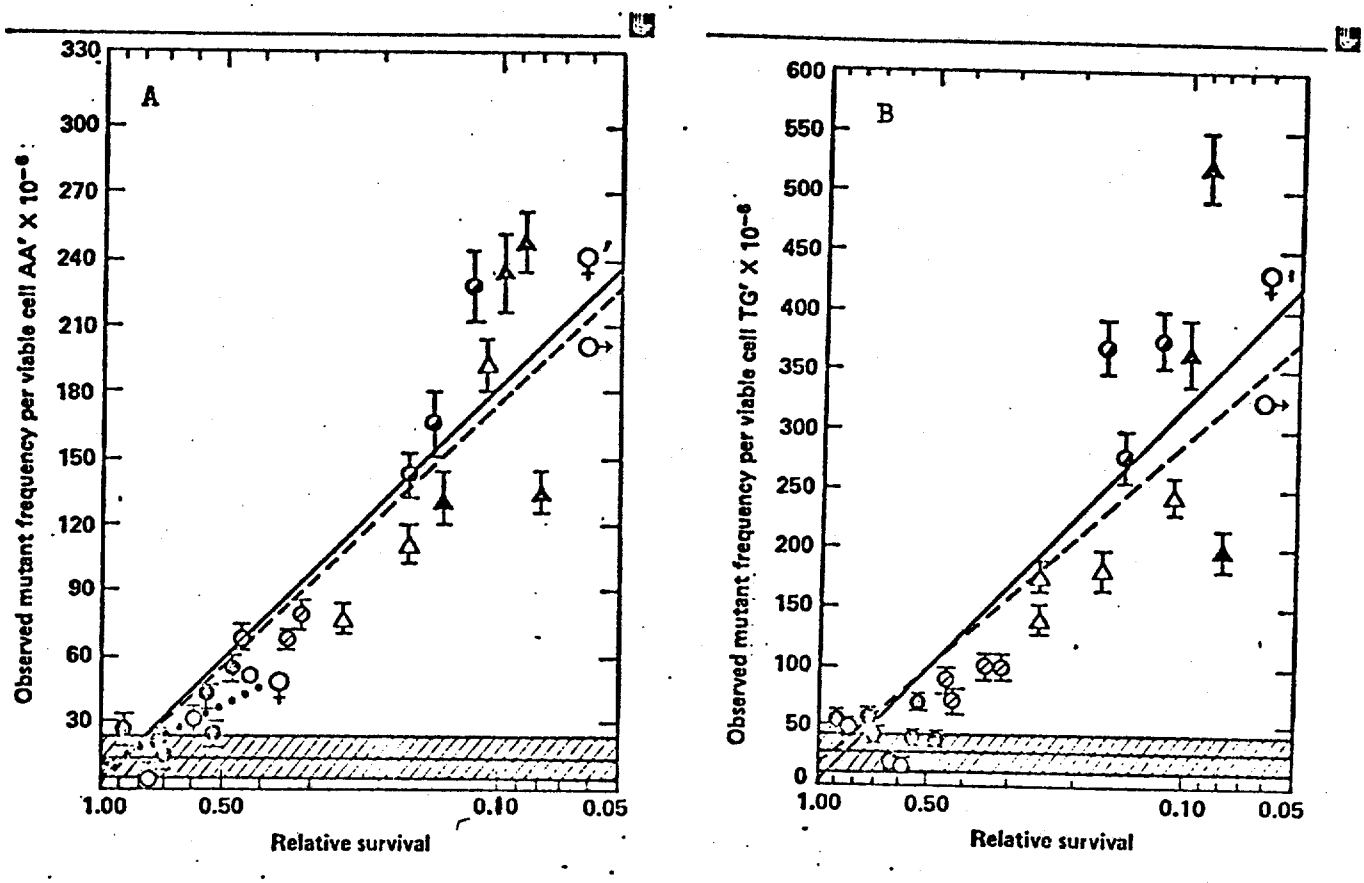


Fig. 6a. Mutant frequencies at multiple loci (aprt, AA^r; hgprt, TG^r) induced by DMN (60 to 100 mM) which was activated for 2 h in the presence of microsomes from male kidney (---△---, 0.5 mg/ml; ---▲---, 1.0 mg/ml) or female kidney (...○..., 0.5 mg/ml; ...●..., 1.0 mg/ml) or testosterone-treated female kidney (—⌀—, 0.5 mg/ml; —⌀—, 1.0 mg/ml). The linear fit for survivals 10% has the following parameters (y = a + bx):

Panel A, AA^r: male, $a=1.54 \times 10^{-6}$, $b=1.78 \times 10^{-4}$;
treated female, $a=1.76 \times 10^{-6}$, $b=1.87 \times 10^{-4}$;
female, $a=8.24 \times 10^{-6}$, $b=8.65 \times 10^{-5}$.

Panel B, TG^r: male, $a=1.37 \times 10^{-5}$, $b=2.86 \times 10^{-4}$;
treated female, $a=2.27 \times 10^{-6}$, $b=3.28 \times 10^{-4}$;

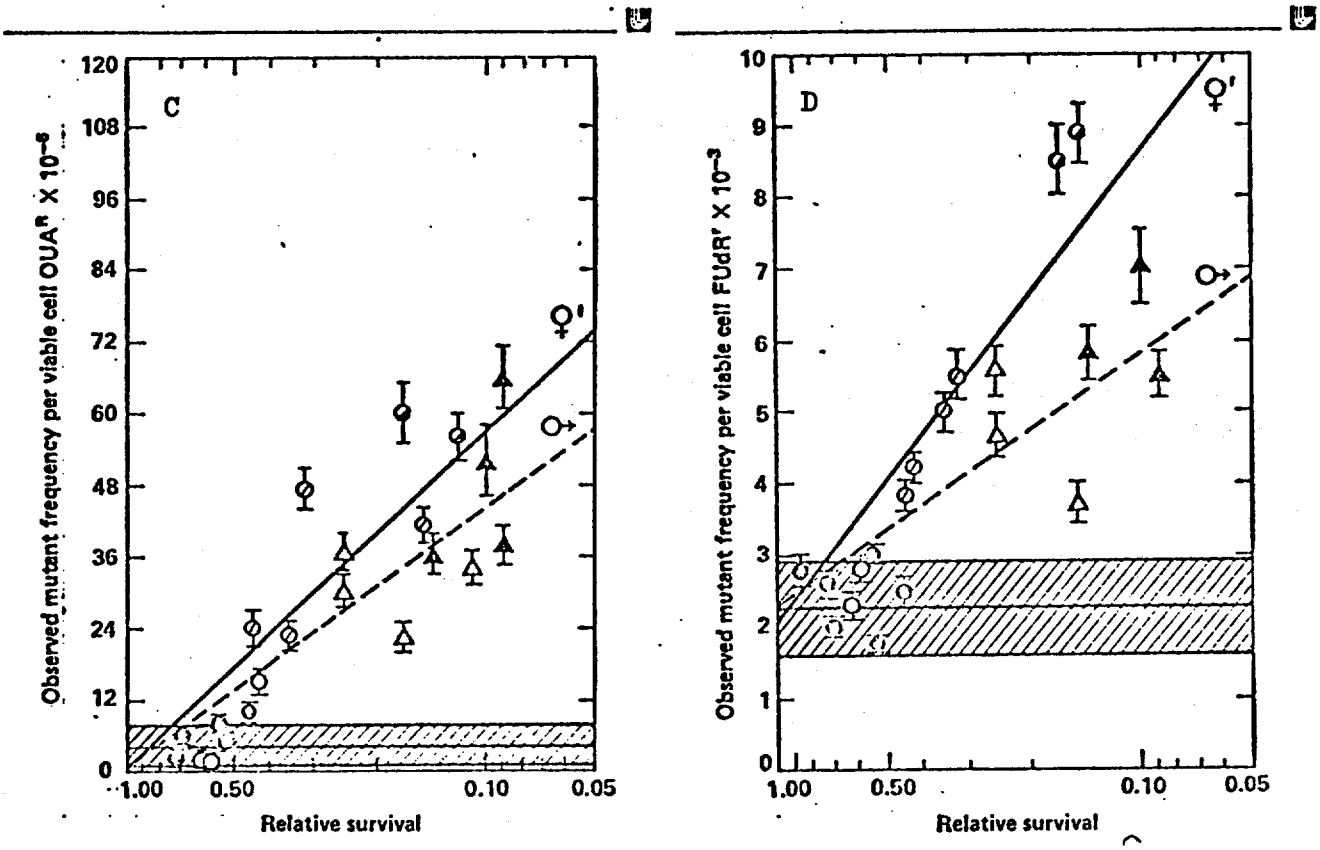


Fig. 6b. Mutant frequencies at multiple gene loci (ATPase, OUA^R; tk, $FUdR^r$) induced by DMN (60 to 100 mM), with all symbols as defined in Fig. 6a. The linear fit is as follows:

Panel C, OUA^R: male, $a=8.57 \times 10^{-7}$, $b=4.39 \times 10^{-5}$;
treated female, $a=6.10 \times 10^{-7}$, $b=5.73 \times 10^{-5}$

Panel D, $FUdR^r$: male, $a=2.27 \times 10^{-3}$, $b=3.61 \times 10^{-3}$;
treated female, $a=2.00 \times 10^{-3}$, $b=6.77 \times 10^{-3}$

general, S-9 activation seems to be sufficient, but microsomal activation (which may represent concentrated toxification enzymes and decreased levels of detoxification enzymes) may be more effective for some compounds, e.g., BP. The cell-mediated techniques fail to activate some compounds and are generally not practical for large-scale mutagen testing; however, assays using feeder layer activation may be able to detect lower levels of mutagen. This is shown in Figs. 3a and 3b, where the SHE-mediated assay detects BP at 2 μg/ml; comparable levels of mutants induced by the microsomal activation of BP (0.3 to 0.75 mg/ml microsomal protein) required a BP concentration of 10 μg/ml. Ongoing experiments show that significant mutant frequencies induced by BP at 10 μg/ml can be detected with S-9 concentrations of ~0.8 mg/ml, but microsomal activation is more efficient. Mouse kidney microsomes are generally less efficient than Aroclor-induced rat liver, the kidney microsomes requiring 100 mM DMN to yield significant mutant frequencies compared to 10 mM in assays using the liver microsomes.

In experiments designed to test the feasibility of combining the MMMA with activation from species and organs other than rat liver, the sex and tissue differences in a steriod-inducible mouse kidney microsomal system were studied. In the C3H/HeJ mouse, male susceptibility to DMN-induced kidney tumors is higher than that of the female; the risk for DMN-induced liver tumors is similar for both sexes. Kidney microsomes from male and female C3H/HeJ mice were compared for their ability to activate DMN into mutagenic metabolites. The mutagenic response was measured at the four drug-resistance marker in CHO cells. At survivals $\geq 10\%$, microsomal preparations from male kidney yielded DMN-induced mutant frequencies approximately 10 to 22 times higher at three of the four loci (about 3 fold for $FUdR^r$) than control or female values. At survivals of at least 10%, kidney preparations from testosterone-treated females increased the DMN-induced mutation at the four loci from 4 to 20 times over that of control and nontreated females. These experiments have shown that the observed mutagenic response from DMN activated by mouse kidney microsomes correlates with known sex and organ specificity observed in whole animal tumor studies, where DMN was activated in vivo. Furthermore, unlike microbial assays where the female activity was

essentially zero, one of the loci (aprt) in the MMMA has a slope significantly greater than zero, suggesting a possible base level of mutagenic activity in female kidney microsomes. The data demonstrate the utility of the MMMA to be coupled with activating material from sources other than rat liver and show the feasibility of relating *in vitro* mammalian mutagenesis to known sex- and organ-specific carcinogenesis *in vivo*.

III. CURRENT AND FUTURE ACTIVITY

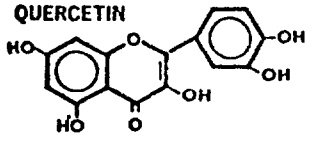
To determine the versatility of the Multiple-Marker Mutagenesis Assay (MMMA), we are testing a variety of diversified agents for their ability to damage specific areas of the genome and thus show specificity for one or more of the four genetic loci evaluated for mutation. If loci differ in their susceptibility to genetic damage by various classes of compounds, then the usefulness of the multiple locus concept will be demonstrated.

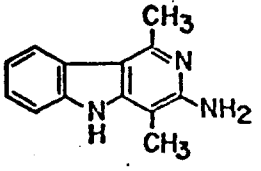
We are currently testing three compounds that are representative of agents found in human food supplies and are known to be potent mutagens in the Salmonella assay. All require activation for full activity and are frameshift mutagens. They induce a type of lesion that we have not heretofore measured in the MMMA. Many hundreds of different flavones and flavonols occur in food-yielding plants; the widely-distributed plant flavonol, quercetin, is mutagenic in the Ames test (MacGregor and Jurd, 1978) and transforms hamster embryo cells (Umezawa et al., 1977). The compound (see Fig. 7) is mutagenic without in vitro activation, but the mutagenic potency is greatly enhanced by the addition of rat liver S-9 fraction. Interestingly, quercetin (with two OH groups in the B-ring) is apparently activated by soluble enzymes present in the supernatant of 100,000 x g centrifugation of S-9. Thus, S-9 activates the compound but presumably concentrated microsomes would not (Brown and Dietrich, Mutat. Res. in press). Two additional compounds are being tested -- Trp-P-1 and Trp-P-2, amino-γ-carboline derivatives that are potent frameshift mutagens in Salmonella and have been identified as pyrolysate products from tryptophan that may be present in charred food (Sugimura, et al., 1977). Both of these compounds (Fig. 7) require metabolic activation and transform hamster embryo cells in vitro (Takeyama, et al., 1977).

In addition to the above compounds, unknowns from the ICI/MRC/NIEHS Collaborative Study are being tested in preliminary toxicity experiments. We will be testing a minimum of ten unknowns in a blind study; the identification of the agents will be disclosed in a workshop to be held in late 1979. The unknowns are made up of a representative carcinogen and noncarcinogen from each of the major classes of carcinogens; in most cases, the noncarcinogens are structural analogues

of more active compounds. This collection of "paired compounds" will allow us to test under controlled conditions the potential for differential sensitivity among the four genetic loci included as markers in the MMMA. The collaborative effort will also allow a systematic comparison between the MMMA and other in vitro analyses for point mutation, DNA repair, mitotic recombination and gene conversion, sister chromatid exchange, and cytogenetic damage.

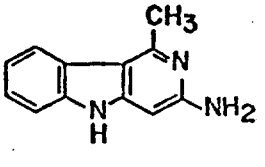
Exposure of a promutagen to the complex cytochrome P-450 metabolizing system in vitro (or to metabolically competent feeder cells) results in several biochemical conversions; the primary metabolite is quickly converted to secondary and tertiary forms. The efficiency of various activation systems will be compared in experiments using compounds selected from the anthracene series, e.g., anthracene, benzanthracene, dibenz(a,c)anthracene, dibenz(a,h)anthracene, and 9,10-dimethyl-1,2-benzanthracene; this series is metabolized by the same microsomal monooxygenase system, collectively known as the cytochromes P-450, as is benzo(a)pyrene. Moreover, the anthracene compounds represent a graded series (from nonmutagenic to very mutagenic) as measured by mutation in the Ames test or whole animal carcinogenesis test. These experiments are designed to compare the relative efficiency of microsomal vs cell-mediated activation and also to test whether our system will yield data at the four gene loci that are proportional to the relative potency of these compounds in other in vitro and in vivo assay systems.





$C_{13}H_{13}N_3$

Trp-P-1



$C_{12}H_{11}N_3$

Trp-P-2

Fig. 7. Chemical structures of (a) a plant flavonol and (b and c) the proposed mutagenic components of pyrolyzed tryptophan.

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